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Dietary Sulfur Amino Acid Effects on Fasting Plasma Cysteine/Cystine Redox Potential in Humans

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Abstract

Objective—Oxidation of plasma cysteine/cystine (Cys/CySS) redox potential (E_hCySS) has been associated with risk factors for cardiovascular disease in humans. Cys and CySS are derived from dietary sulfur amino acids (SAA), but the specific effects of SAA depletion and repletion on Cys/CySS redox indices are unknown. The present study examined the effect of dietary SAA intake level on free Cys, free CySS and E_hCySS in human plasma under fasting conditions.

Research Methods and Procedures—Healthy individuals aged 18–36 y (n=13) were equilibrated to foods providing the RDA for SAA and then fed chemically defined diets without SAA (0 mg·kg^{-1}·d^{-1}; n=13) followed by SAA at levels approximating the mean (56 mg·kg^{-1}·d^{-1}; n=8) or 99th percentile (117 mg·kg^{-1}·d^{-1}; n=5) intake levels of Americans. Fasting plasma samples were collected daily during 4-d study periods and analyzed for free Cys, free CySS and the E_hCySS.

Results—The SAA-free diet significantly ($p<0.05$) decreased plasma free Cys concentrations and oxidized E_hCySS values after 4 days of SAA depletion. With SAA repletion at 56 mg·kg^{-1}·d^{-1}, plasma free Cys increased significantly and values for E_hCySS became more reducing. Administration of a diet providing a higher dose of SAA (117 mg·kg^{-1}·d^{-1}) resulted in a significantly higher level of free Cys and a more reducing E_hCySS.

Conclusions—These results show that free Cys and Cys/CySS redox potential (E_hCySS) in fasting plasma are affected by dietary SAA intake level in humans. Significant changes occur slowly over 4 days with insufficient SAA intake, but rapidly (after 1 day) with repletion.

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Role of each author in work: DJ and TZ conceived and designed the study; NG, CA and TZ provided oversight for the study; NG, YP and YL conducted the study and collected the data; YP and TY provided statistical analysis; DJ, YP, CA and TZ drafted and revised the paper; DJ, YP and TZ had primary responsibility for approval of final manuscript.

Keywords

Oxidative stress; amino acid balance; cardiovascular disease; methionine

Introduction

Proteins contain Cys residues which readily undergo reversible oxidation-reduction reactions. In vitro studies show that controlled variation in extracellular thiol/disulfide redox state alters cell signaling of monocyte adhesion to endothelial cells [1], platelet activation [2], cell proliferation [3,4] and apoptosis [5] apparently mediated through integrins, metalloproteinases, growth factor receptors and ion channels. In vivo studies in humans show that oxidized thiol/disulfide redox potential is associated with increased carotid intima media thickness [6], decreased flow mediated dilation [7], reversible myocardial perfusion defects [8] and persistent atrial fibrillation [9]. Thus, the dietary factors affecting extracellular thiol/disulfide redox potential in human plasma could be important in cardiovascular disease.

The predominant low molecular weight thiol/disulfide system present in plasma is cysteine/cystine (Cys/CySS). CySS concentration in human plasma is considerably higher than Cys concentration under most conditions [10–12], and accumulating evidence indicates that the balance between Cys and CySS could be an important health determinant. Because the stoichiometry is 2 Cys per CySS, the balance is often expressed in terms of the redox potential (E_hCySS). The reducing force of the plasma Cys/CySS couple is quantitatively expressed by its redox potential (E_hCySS), related to Cys and CySS concentrations by the Nernst equation [13]. E_hCySS in healthy individuals aged 25–35 y was found to be -80 ± 9 mV [10], and cross-sectional studies show that this value is more positive (oxidized) in association with aging [14], cigarette smoking [15], chronic alcohol abuse [16] and anticancer therapy [17]. Increased plasma CySS concentration and/or oxidized plasma E_hCySS have been associated with human disease risk, e.g., persistent atrial fibrillation [9], peripheral vascular disease [6] and age-related macular degeneration [18].

In a study of dietary intake of SAA in rats, we found that SAA-deficient food (providing no Cys and 17% of control diet SAA intake as Met) resulted in oxidation of plasma E_hCySS, with corresponding changes in gut GSH/GSSG redox potentials and cell growth indices [19]. A study of diurnal variation of GSH and Cys in human plasma showed that plasma E_hCySS varied in an apparent meal-related pattern [20]. E_hCySS was maximally oxidized at early hours of the morning (0430–0630 AM), became transiently reduced 2–3 h after each meal, and was maximally reduced at 2030, which was 3 h after the largest meal [20]. Together, these data suggest that in humans, E_hCySS could vary as a consequence of dietary intake of SAA.

The present study was designed to determine the effects of dietary SAA depletion and repletion on the fasting plasma cysteine/cystine redox potential in humans. The study used a semisynthetic, chemically defined diet design based upon the studies of Young and coworkers [21,22], which allows specific changes in L-amino acid content. To minimize contributions of other environmental factors which could affect oxidative stress and thereby alter E_hCySS, fasting plasma levels were obtained at a consistent time in the controlled setting of a metabolic research unit.

Materials and methods

Materials

Sodium heparin, bathophenanthroline disulfonate sodium salt (BPDS), sodium iodoacetate, dansyl chloride, L-serine, Cys, CySS, and sodium acetate trihydrate were from Sigma Chemical Corp. (St. Louis, MO, USA). γ-Glutamylglutamate (γ-Glu-Glu) was from MP Biomedicals Corp. (Irvine, CA, USA). Boric acid, sodium tetraborate, potassium tetraborate, perchloric acid, and acetic acid were reagent grade and purchased locally. Methanol, acetone, and chloroform were HPLC grade.

Subjects

This study was reviewed and approved by the Emory Investigational Review Board. A total of 13 volunteers, self-described as healthy, were recruited beginning January 1, 2005 by posting fliers in public locations in the Atlanta/Emory University community. Following informed consent, all subjects were screened in the outpatient unit of the Emory University Hospital GCRC, where a medical history and physical examination, body height and weight, fasting standard blood chemistry and hematology tests and a urinalysis were performed (a serum pregnancy test was also performed in females). Indirect calorimetry was used to determine resting energy expenditure (REE). Eligibility was established by the absence of evidence of acute or chronic illness, no current smoking history, and a body mass index (BMI) < 30.

Within one month following screening, the subjects were scheduled to begin the study. Subjects taking antioxidants, nutrient supplements (with the exception of once-daily multivitamin-mineral supplements) or acetaminophen were asked to discontinue these two weeks prior to the onset of the studies. A 3-day equilibration period was used to normalize the 13 subjects with regard to diet (Figure 1). During equilibration, the standardization diet was based on the U.S. Recommended Dietary Allowances [23] providing maintenance energy and protein intake, approximating the RDA for SAA intake (12.2 mg/kg body weight Met per day plus 6.6 mg/kg body weight Cys per day). During the subsequent study periods, the subjects were given chemically defined oral diets providing maintenance energy intake and 1.0 g/kg body weight/day protein equivalents as an L-amino acid mixture (Figure 1, see below). All study meals were prepared in the GCRC metabolic kitchen, given at standardized mealtimes and consumed over 20 min, and intake was monitored by the GCRC Bionutrition Unit staff. The study period consisted of two consecutive 4-day study periods. During the first phase, all subjects received a chemically defined diet devoid of Met and Cys (SAA depletion phase). This period was followed by an SAA repletion phase which was isocaloric based upon gross energy content and isonitrogenous with the chemically defined diets providing either 56 mg/kg body weight SAA per day (n=8) or 117 mg/kg body weight per day (n=5) respectively (Figure 1). The chemically defined diets contained a distribution of Met:Cys of 2:1 by gravimetric weight. The intake of 56 and 117 mg/kg body weight SAA per day levels approximate the mean and 99th percentile daily intake of SAA in the American diet, respectively, as determined in the National Health and Nutrition Examination Survey (NHANES III) [23]. Overnight fasted plasma was obtained at 0830 AM for study endpoints.

Diet and nutrient intake

The protein equivalents of diets were supplied in the same forms of specific L-amino acid mixtures (Ajinomoto USA, Teaneck, NJ), providing 1.0 g/kg body weight per day as outlined in detail [21,22]. The standard mixture was patterned after hen’s egg protein and provides all 9 indispensable (essential) amino acids, including Met, in amounts sufficient for the mean requirements of healthy young adults [21,22], but which are higher than the

requirements proposed by the World Health Organization [21,24]. The standard amino acid mixture also contained 9 dispensable (non-essential) amino acids, including Cys and glutamate, and was glutamine- and taurine-free. To compensate for the difference in Met + Cys between the 0, 56 and 117 mg/kg body weight per day SAA diets, the amount of all non-essential amino acids were proportionally changed to maintain a constant dietary nitrogen content while at the same time maintaining them as isoenergetic. Similarly, for all diets, the proportion by gravimetric weight of Met:Cys was constant (2:1). To improve palatability, a powdered flavoring agent was added to the liquid amino acid mixture (provided as a sherbet-based drink) (19,20). The Cys was added immediately prior to consumption to minimize Cys oxidation to CySS prior to consumption. The dietary energy (1.3 times measured REE) was mainly derived from lipid and carbohydrate sources provided in the form of protein-free wheat starch and butter/safflower oil cookies and a sherbet-based drink, as outlined [21,22]. All subjects were completely compliant with all research meals as verified by the GCRC bionutrition staff.

Adequate oral hydration and vitamin, mineral and electrolyte requirements were provided to all subjects to meet or exceed recommended allowances [21]. Water was provided *ad libitum* with monitoring to ensure urine output of at least 700 mL during each 24-h period. All subjects received on a daily basis one multivitamin-multimineral capsule with iron (One-A-Day; Miles Inc., Elkhart, IN); three potassium tablets (K-LYTE;20 meq each, generic); four calcium tablets (TUMS; SKB Corp., Pittsburgh, PA); two sodium chloride tablets (1 gram tablets; Eli Lilly and Co., Indianapolis, IN); two choline capsules (250-mg; Lee Nutrition Inc., Cambridge, MA); and one magnesium oxide tablet (400 mg). All supplements were administered on a regular schedule by the GCRC research nurses. Stool softener (oral docusate) was provided as needed to a few subjects. Body weights were determined daily and vital signs were obtained every 8 h. Low-level activity was allowed and restricted to walking on the GCRC.

Sampling and redox analyses of E_hCyS and E_hGSSG

Blood was collected at 0830 AM each day of the depletion and repletion period before the morning meal being provided using a standardized and validated procedure for the further analyses of free Cys, CySS, GSH, and GSSG. The blood (1.35 mL) was collected via a heparinized 5 mL syringe with a butterfly needle and immediately transferred to a microcentrifuge tube containing 0.15 mL of preservation solution designed to alkylate Cys and prevent its oxidation. The preservation solution included 0.504 mol/L L-serine, 9.3 mmol/L batho-phenanthroline disulfonic acid disodium salt(BPDS), 0.165 mol/L γ-glutamylglutamate, 0.401 mol/L boric acid, 0.144 mol/L iodoacetic acid, and 2.5 mg (per mL) heparin. The BPDS, a chelator, was added to minimize autoxidation. The serine and borate were included to inhibit γ-glutamyltranspeptidase and to buffer pH to a value where iodoacetate rapidly carboxymethylated thiols. These samples were immediately centrifuged in a microcentrifuge at 13,000 g for 30 s, and 200 μL of plasma was aliquoted to a tube containing 10% ice-cold perchloric acid and 0.2 mol/L boric acid solution. The entire sampling procedure took less than 2 min for each sample, avoiding artifacts from delays in processing [12]. Samples were stored at −80° C and analyzed within 1 month. Stability tests showed that samples are stable under these conditions for at least 2 months [25].

A 300-μl aliquot of each supernatant was transferred to a fresh microcentrifuge tube. The pH was adjusted to 9.0 ± 0.2 with a KOH/tetraborate solution. After 20 min, 300 μl of the dansyl chloride solution was added, and the samples were mixed and placed in the dark at room temperature for 16 to 26 h. Chloroform (500 μl) was then added to each tube to extract the unreacted dansyl chloride, and samples were assayed by HPLC [25].

E_hCySS was calculated using the Nernst equation, $E_h = E_o + RT/nF \ln[CySS]/([Cys]^2)$, where E_o is the standard potential (−250 mV for CySS/Cys at pH 7.4), R is the gas constant, T is the absolute temperature, n is 2 for the number of electrons transferred, and F is Faraday's constant [10]. Protein cysteinylation was found to correlate with plasma CySS and was not measured in the current study [10]. In previous studies of subjects with both normal homocysteine and homocysteinemia, total free and protein-bound homocysteine have been shown to correlate [26]. In the current study, study subjects were not screened for homocysteinemia. E_hGSSG was calculated as above using respective GSH and GSSG concentrations and an E_o of −264 mV for pH 7.4.

Statistics

Minitab software (version 15; Minitab, Inc, State College PA) was used for all analyses. The protocol was designed so that each individual was studied without SAA (depletion) and with either 56 or 117 $mg \cdot kg^{-1} \cdot d^{-1}$ (repletion). With this design, all 13 subjects were treated identically for the first study period but differed in dose for the second study period. The fasting morning blood sample immediately following the 3-day equilibration phase represented the baseline sample for the SAA depletion period (day 0). The subsequent four fasting morning samples represented the SAA depletion phase (depletion days 1–4; Figure 1). The fasting morning blood sample following the SAA depletion represented the baseline timepoint for the SAA repletion phase (day 0 of repletion phase). The subsequent four fasting morning samples represented the SAA repletion phase (repletion days 1–4; Figure 1). One-way ANOVA was used to evaluate effects of time on plasma Cys, CySS and E_hCySS in all 13 subjects during SAA depletion. One way ANOVA was also used to assess the effects of SAA repletion of redox indices within the lower SAA repletion dose group (n = 8 for the 56 $mg \cdot kg^{-1} \cdot d^{-1}$). To assess possible SAA repletion dose-responses, we performed two-way repeated measures ANOVA to compare the repletion data between the 8 subjects receiving SAA repletion at 56 $mg \cdot kg^{-1} \cdot d^{-1}$ versus the 5 subjects receiving SAA repletion at 117 $mg \cdot kg^{-1} \cdot d^{-1}$. Tukey simultaneous tests were used for post-hoc comparisons. Data are expressed as mean ± SEM. Results were considered significant at $P \leq 0.05$.

Results

Study subject characteristics

Thirteen subjects aged 18 to 36 y were studied, eight in Group 1 with 56 mg/kg body weight SAA (18.7 mg/kg Cys) and five in Group 2 with 117 mg/kg body weight SAA (39 mg/kg Cys) daily intake (see Table 1). The 13 subjects included 8 males and 5 females, with 6 Whites, 5 African-Americans, 1 Asian and 1 other. Groups 1 and 2 did were not different in mean age (± SEM) at 23.6 ± 2.7 y and 24.9 ± 2.2 y, respectively. BMI ranged from 20 to 26 with mean (± SEM) values of 22.5 ± 1.8 and 22.5 ± 1.5, for Groups 1 and 2, respectively, and were not statistically different. Subjects reported no acute or chronic illness and none were taking regular prescription medications.

Effect of SAA depletion on plasma free Cys, free CyS and E_hCyS

At baseline (following equilibration with the 18.7 $mg \cdot kg^{-1} \cdot d^{-1}$ SAA diet), the mean fasting Cys value was 10.6 ± 0.6 μmol/L for all 13 subjects (Fig 2A). The free Cys concentrations by one-way ANOVA decreased with time during SAA depletion phase (Fig 2A; $p < 0.05$) with the minimum value occurring after 4 days (7.6 ± 0.4 μmol/L). The free Cys values were significantly different from baseline on days 2 to 4 by post-hoc testing (Fig 2A). The data show a gradual plasma free Cys decrease occurs with an approximate rate of 0.6 μmol/L per day.

The free CySS concentration in fasting plasma at baseline was (74 ± 5 μmol/L). Plasma free CySS concentrations did not change significantly over time in subjects fed the SAA depleted diet (Fig 2B).

Fasting plasma E_hCySS was −75 ± 2 mV (n = 13) at baseline. This value is similar to the previously reported mean values of −72 to −77 mV for fasting samples taken between 06:30 AM and 08:30 AM in a diurnal variation study [20]. E_hCySS became significantly more positive (less reducing) during the SAA-depletion period, with values at day 4 (−67 ± 2) significantly more oxidized than baseline and depletion day 1 values by post-hoc testing ($p < 0.05$) (Fig 2C).

Effect of SAA repletion on plasma free Cys, free CyS and E_hCyS

The effects of dietary SAA repletion on plasma Cys/CySS redox was determined in 8 of the 13 subjects studied above. These individuals were fed isoenergetic, isonitrogenous, chemically defined diets providing SAA intake approximating the mean American intake (56 $mg \cdot kg^{-1} \cdot d^{-1}$) from NHANES III) [27]. Figure 3 shows the free Cys values in fasting plasma for these 8 subjects during their SAA repletion phase. SAA at this dose significantly increased plasma free Cys concentrations over time (Figure 3A; $P<0.005$), with values rising significantly above the post-depletion baseline values by day 1 of SAA repletion and then plateauing by day 2 of repletion. Plasma free CySS levels did not change during SAA repletion (Figure 3B). However, due to the increase in plasma free Cys, the E_hCySS became more reducing during repletion over time from the post-depletion baseline value of −70 ± 3 mV ($P<0.05$) (Figure 3C). The most reduced values occurred by day 2 of SAA repletion (−81 ± 2 mV; $P < 0.05$ versus baseline).

SAA repletion dose-responses

To explore potential dose-response effects of SAA repletion on the free Cys, free CySS and E_hCySS in fasting plasma, we compared the repletion data between the 8 subjects receiving SAA repletion at 56 $mg \cdot kg^{-1} \cdot d^{-1}$ and the 5 subjects receiving SAA repletion at 117 $mg \cdot kg^{-1} \cdot d$. Two-way repeated measures ANOVA were performed to determine the dose response of SAA on free Cys, free CySS and E_hCySS. This repeated measures ANOVA showed the increase in plasma free Cys concentrations over time with the higher SAA repletion dose was marginally significant compared to the lower dose ($p=0.055$) (Figure 4A). In contrast, plasma free CySS levels were not statistically different with the lower versus the higher SAA repletion doses (Figure 4B). Redox potential (E_hCySS) for the Cys-CySS couple was significantly affected by SAA repletion dose; the values for E_hCySS were more reducing with the 117 $mg \cdot kg^{-1} \cdot d$ SAA repletion dose over time compared to the 56 $mg \cdot kg^{-1} \cdot d^{-}$ dose (Figure 4C).

Effects on free GSH redox couple in fasting plasma

For all conditions described, analyses were also performed to determine free GSH, free GSSG and the redox potential for the GSH/GSSG couple (E_hGSSG). These data showed the GSH and GSSG concentrations were comparable to those obtained previously for baseline of fasting morning conditions (GSH: 1.6 ± 0.2 μmol/L, GSSG:0.08 ± 0.03 μmol/L expressed as mean ± SEM) [25]. The plasma free GSH did not change during depletion and repletion period (Figure 5). No significant effect of SAA intakes on GSSG or E_hGSSG in fasting plasma was detected (data not shown).

Discussion

Accumulating evidence indicates that plasma GSH/GSSG and Cys/CySS pools could be important in disease risk in humans and be especially relevant to cardiovascular disease as

possible biomarkers of risk. For instance, both E_hGSSG and E_hCySS were found to be oxidized in association with age [14], cigarette smoking [15] and increased BMI [7]. E_hGSSG was also found to be oxidized in association with type 2 diabetes [28] and alcohol abuse [16], and E_hCySS was oxidized in association with increased proinflammatory cytokines, IL-1β and TNF-α [29], and reduced in individuals consuming a Mediterranean diet [30]. The concentration of CySS, which is a co-variable with E_hCySS, is also increased in association with TNF-α [29], endothelial dysfunction [7], and increased carotid intima media thickness [6]. In addition, a substantial literature shows that dietary inducers can affect tissue levels of GSH and related metabolites [31]. Thus, there is a need to understand dietary factors which affect plasma E_hGSSG, E_hCySS or CySS concentration in humans.

The present study addressed whether recent history of dietary sulfur amino acid intake affected fasting plasma E_hGSSG, E_hCySS or CySS concentration. The data show no significant effects on E_hGSSG or CySS concentration. However, the results show that low SAA intake decreased fasting blood levels of free Cys and resulted in an oxidation of E_hCySS over a period of 4 days. In contrast, 1 day with SAA at the mean American intake level was sufficient to restore the free Cys and E_hCySS in fasting plasma. These data indicate that if fasting plasma E_hCySS is used for assessment of cardiovascular disease risk, the previous 24-h intake of SAA could mask oxidation due to a long-term inadequate SAA intake. On the other hand, surveillance data show that long-term inadequate SAA intake is not common [27], and 1 day of inadequate SAA intake is not sufficient to result in a falsely oxidized E_hCySS value. Thus, although variations in recent history of SAA intake can affect E_hCySS, this effect is insufficient to seriously compromise use of E_hCySS as a biomarker. The lack of effects of SAA intake on fasting plasma E_hGSSG and CySS concentration indicate that these parameters are minimally affected by the recent history of SAA intake.

In vitro studies indicate that oxidation of plasma E_hCySS could be mechanistically important in disease development. While plasma E_hGSSG is thought to indicate tissue oxidative stress, E_hCySS appears to reflect extracellular oxidative processes [14]. A more oxidized E_hCySS in culture media was found to increase sensitivity of retinal pigment epithelial cells [5] and endothelial cells (Y-M Go and DP Jones, unpublished observation) to apoptosis, enhance binding of monocytes [1,32] and neutrophils [33] to endothelial cells and increase production of proinflammatory cytokines in monocytes [29]. At least some of these effects are mediated through cell surface thiols [1], indicating that oxidation of plasma thiol/disulfide couples could directly affect function of extracellular or cell surface proteins, including receptors, integrins and metalloproteinases [34].

The magnitudes of the diet-induced changes after 4 d further suggest that fasting plasma E_hCySS could be mechanistically important in cell signaling through cell surface protein dithiol/disulfide couples; an 8 mV oxidation in E_hCySS (as observed after 4 days of SAA depletion) is equivalent to an approximately 2-fold change in protein dithiol/disulfide ratio while an 18 mV change (difference between 4 d with high SAA versus no SAA) is equivalent to a 4- fold change [35]. The magnitude of the E_hCySS oxidation observed with SAA depletion is similar to differences associated with aging [14,18], smoking [15], or alcohol consumption [16]. Also, the mean difference in E_hCySS for individuals with high and low risk of persistent atrial fibrillation was 10 mV [9]. The similarities in magnitude of E_hCySS in these disorders and with SAA depletion in the present study suggest that diet-induced changes at extreme levels of SAA intake may have pathophysiologic effects.

Deficient intake of SAA could have effects other indirect effects. For instance, taurine, a product of Cys oxidation, was found to protect against hyper-homocysteinemia-induced toxicity apparently through an antioxidant mechanism [36]. Insufficient intake could also have effects through altered transport of Cys and CySS in vivo because the transporters for

these amino acids are common to other amino acids. Changes in SAA would thereby affect the balance of amino acids required for cellular protein synthesis. Furthermore, the GSH turnover is decreased by SAA intake [21,22], indicating that transport of precursors could be limiting. In vitro studies show that culture of HT29 cells in Cys- and CySS-free media results in oxidation of the cellular E_hGSSG and increased GSH synthesis upon re-addition of the amino acids to the culture media [11]. The x_c^- system functions in uptake of CySS and is transcriptionally induced through the Nrf-2 transcription factor by oxidative conditions [11]. Thus, it appears likely that altered transport occurs in response to SAA insufficiency.

The present study of healthy, young individuals does not allow conclusions concerning older individuals who are more at risk of disease. The present study included young, healthy individuals, but clinical data for E_hCySS and disease largely include middle age and older individuals. Some of these latter studies provide stronger associations of disease risk with the free CySS concentration than with E_hCySS [6,37,38]. Thus, experiments are warranted to determine whether similar or greater effects on the free CySS and E_hCySS occur in older individuals and in individuals with disease risk.

In conclusion, the data show that for young adults with previously sufficient SAA intake, the free Cys, free CySS and E_hCySS in fasting plasma are affected by SAA intake over a 4-day period. The results show that SAA repletion rapidly improves Cys/CySS redox status (within 24 h) after a diet devoid of methionine, cysteine or cystine. The magnitude of differences between intake of insufficient and sufficient diets were similar to previously reported differences associated with aging, oxidative stress and disease, suggesting that SAA intake at extreme levels could contribute to disease processes associated with these redox parameters.

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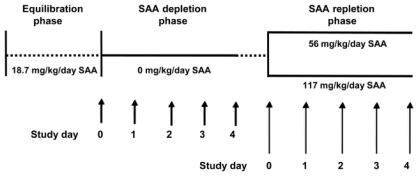

Fig 1. Study scheme for dietary sulfur amino acid (SAA) effects on plasma cysteine, cystine and cysteine/cystine redox potential

The 13 healthy adult study subjects were each admitted into the Emory General Clinical Research Center (GCRC) for a 13-day inpatient study period. The first 3 days were an equilibration phase, during which conventional food items approximating the RDA for SAA (18.7 $mg \cdot kg^{-1}$) were given. The subjects were given chemically defined oral diets for a subsequent 10-day study period providing maintenance energy intake and 1.0 g/kg body weight/day protein equivalents. During the study period without SAA, all 13 subjects were fed a chemically defined diet devoid of Met and Cys (SAA insufficiency phase). This was followed by a SAA repletion phase during which the chemically defined diet providing either 56 mg SAA per kg body weight per day (n=8), similar to average SAA intake based on U.S. consumption data, or 117 $mg \cdot kg^{-1} \cdot d^{-1}$ SAA (n=5), similar to 99 percentile intake of Americans, was given. Protein equivalents were provided as an L-amino acid mixture containing 9 essential amino acids, without or with methionine (Met) and 9 non-essential amino acids, without or with cysteine (Cys). The SAA repletion diets were made isocaloric and isonitrogenous to the SAA insufficient diet by proportional adjustment of the 9 non-essential amino acids in the L-amino acid mixture. Arrows designate time points for overnight fasting plasma sample collection for free Cys, free CySS and E_hCySS, which were obtained at 0830 AM.

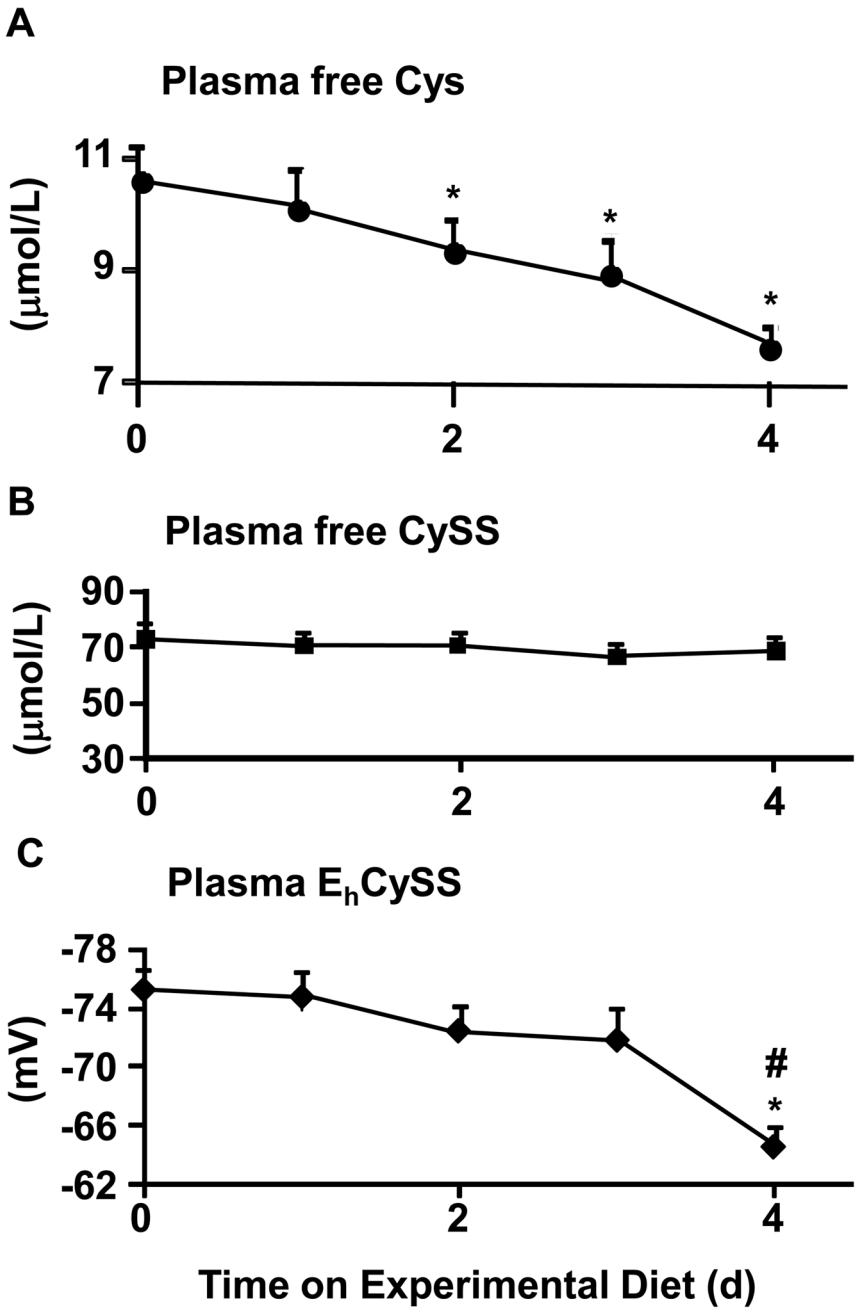

Fig 2. The free cysteine (Cys), free cystine (CySS) and Cys/CySS redox potential (E_hCySS) in human plasma under fasting conditions following intake of SAA-free diet

The study protocol is as outlined in Figure 1. Data shown are derived from fasting plasma samples (n=8) obtained at morning time points after completion of the equilibration period and for the next 4 days during consumption of diets devoid in methionine, Cys and CySS. **A**. The free Cys in fasting plasma. A significant effect of time was observed by one-way ANOVA ($P<0.05$). *Significant difference from baseline values ($P<0.05$). **B**. The free CySS in fasting plasma. No change over time was observed. **C**. Fasting plasma E_hCySS. A significant effect of time was observed by one-way ANOVA ($P<0.05$). *Significant difference from baseline values and $^{\#}$from day 1 values by Tukey's post-hoc test ($P<0.05$). Data are expressed as mean ± SEM.

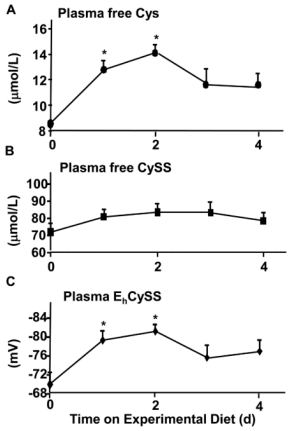

Fig 3. Free Cys, free CySS and E_hCySS in human plasma obtained under fasting conditions following SAA repletion

The study protocol is as outlined in Figure 1. Data shown are derived from fasting plasma samples (N = 8) obtained at morning time points following completion of SAA depletion period and for the next 4 days during repletion with diets providing 56 mg·kg^{-1}·d^{-1} SAA. **A**. The free Cys in fasting plasma. A significant effect of time was observed by one-way ANOVA (P<0.05). *Significant difference from baseline values (P<0.05). **B**. The free CySS in fasting plasma. No change over time was observed. **C**. Fasting plasma E_hCySS. A significant effect of time was observed by one-way ANOVA (P<0.05). *Significant difference from baseline values by Tukey's post-hoc test (P<0.05). Data are expressed as mean ± SEM.

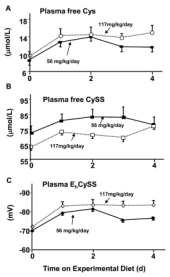

Fig 4. Dose response to SAA repletion on free Cys, free CySS and E_hCySS in human fasting plasma following SAA repletion

The study protocol is as outlined in Figure 1. Data shown are derived from fasting plasma samples obtained at morning time points following completion of SAA depletion period and for the next 4 days during repletion with diets providing either 56 mg·kg^{-1}·d^{-1} SAA (solid lines: n=8) or 117 mg·kg^{-1}·d^{-1} SAA (dotted line: n=5). **A**. The free Cys in fasting plasma. A trend for an effect of SAA dose was observed by two-way repeated measures ANOVA (P=0.055). **B**. The free CySS in fasting plasma. No significant effect of SAA dose was observed by two-way repeated measures ANOVA. **C**. Fasting plasma E_hCySS. A significant effect of SAA dose was observed by two-way repeated measures ANOVA (P<0.05). Data are expressed as mean ± SEM.

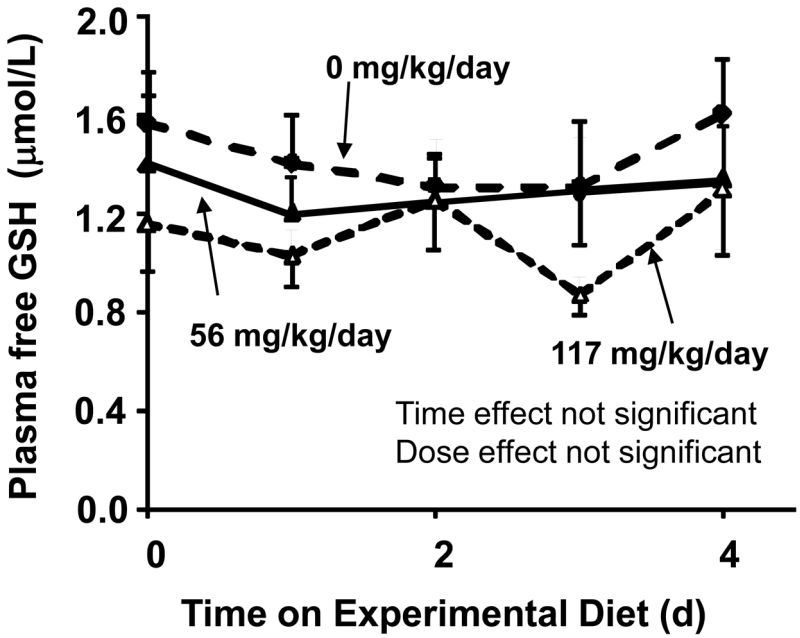

Fig 5. Dose response of SAA repletion on free GSH in human fasting plasma following SAA repletion

The study protocol is as outlined in Figure 1. Data shown are derived from fasting plasma samples obtained at morning time points from baseline (completion of SAA depletion period, closed circle, dotted line) and for the next 4 days during repletion with diets providing either 56 $mg \cdot kg^{-1} \cdot d^{-1}$ SAA (closed triangle, solid lines: n=8) or 117 $mg \cdot kg^{-1} \cdot d^{-1}$ SAA (open triangle, dotted line: n=5). No significant effect of SAA dose was observed by two-way repeated measures ANOVA. Data are expressed as mean ± SEM.

TABLE 1

Characteristics of the study participants

Group 1: Subjects receiving sulfur amino acids at 56 mg·kg^{-1}·d^{-1}			
Age	**Sex**	**Race**	**BMI**
18	F	White	22.5
20	M	White	22.3
21	M	Black	20.7
23	F	Black	24.8
23	M	White	23.2
25	M	Asian	21.0
33	F	Black	26.0
36	M	Black	20.0

Group 2: Subjects receiving sulfur amino acids at 117 mg·kg^{-1}·d^{-1}			
Age	**Sex**	**Race**	**BMI**
18	F	White	26.3
19	M	Other	23.6
21	F	Black	20.3
29	M	White	24.7
31	M	White	25.6